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In silico cytotoxicity assessment on cultured rat intestinal cells deduced from cellular impedance measurements

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ABSTRACT

Early and reliable identification of chemical toxicity is of utmost importance. At the same time, reduction of animal testing is paramount. Therefore, methods that improve the interpretability and usability of *in vitro* assays are essential. xCELLigence's real-time cell analyzer (RTCA) provides a novel, fast and cost effective *in vitro* method to probe compound toxicity. We developed a simple mathematical framework for the *qualitative* and *quantitative* assessment of toxicity for RTCA measurements. Compound toxicity, in terms of its 50% inhibitory concentration IC_{50} on cell growth, and parameters related to cell turnover were estimated on cultured IEC-6 cells exposed to 10 chemicals at varying concentrations. Our method estimated IC_{50} values of 113.05, 7.16, 28.69 and 725.15 μM for the apparently toxic compounds 2-acetylamino-fluorene, aflatoxin B1, benzo-[a]-pyrene and chloramphenicol in the tested cell line, in agreement with literature knowledge. IC_{50} values of all apparent *in vivo* non-toxic compounds were estimated to be non-toxic by our method. Corresponding estimates from RTCA's in-built model gave false positive (toxicity) predictions in 5/10 cases. Taken together, our proposed method reduces false positive predictions and reliably identifies chemical toxicity based on impedance measurements. The source code for the developed method including instructions is available at https://git.zib.de/bzfgupta/toxfit/tree/master.

1. Introduction

Identifying adverse effects of chemicals with respect to their distinguishing temporal and dose-dependent mode of action denotes a formidable challenge in toxicological research. Often, however, identifying toxic effects is rather difficult since indirect and long-term effects on both humans and the environment are not easy to assess, require an immense investment of resources, and moreover, systematic testing itself may be unethical. Consequently, information about the hazards posed by a vast array of chemical substances is lacking (Schoeters, 2010).

A typical test routine for the prediction of toxicity relies on a sequence of stringent *in vitro* assays and *in vivo* experiments. Established mammalian cell-based *in vitro* assays for toxicity testing produce a high number of false positive results (Kirkland et al., 2007). Note, that any positive *in vitro* toxicity result leads to successive animal (*in vivo*) testing for validation. In order to avoid unnecessary animal testing, it is highly desirable to improve the predictive power of *in vitro* toxicity tests. Therefore, it is of utmost importance to integrate novel, resource-efficient approaches into testing strategies, which allow benchmarking the safety levels of substances and guide further experimentations.

The impedance-based xCELLigence real-time cell analyzer (RTCA) is an *in vitro* assay that provides a high-resolution temporal information about the physiological status of the cultivable attached cells such as cell number, morphology and adhesion. Upon treatment of cells with a toxic chemical, the impedance measurements (Fig. S1) change due to perturbation in the underlying cellular processes. Compounds with distinguishing RTCA profiles are therefore indicative of a characteristic mode of action (MoA) and the magnitude of drug effect on the cells (Abassi et al., 2009). The in-built RTCA software (Manual, 2009) uses a sigmoidal dose-response model which is fitted to impedance measurements at a particular time instance. However, a problem with this model is that it may produce small IC_{50}/EC_{50} values (indicating potent effects) even when

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the absolute magnitude of the change in impedance is small and possibly within the error of measurement, producing false positive predictions (Fig. 1). Thus, to delineate the precise relation of cellular response in context of different conditions (such as chemical concentration and time of administration) more reliability, application of mechanistic mathematical modeling is crucial.

Previous studies used clustering-based approaches for screening a library of chemical compounds on the basis of the RTCA profile (Abassi et al., 2009; Xi et al., 2014). Clustering-based methods provide a quick and reliable way of screening chemicals, but limitations are posed by the requirement of large training datasets and the test conditions (e.g. chemical concentrations used). Moreover, these methods may preclude a mechanistic insight into the chemicals' mode of action, as well as a quantitative characterization in terms of a dose-response profile. For a detailed insight into the chemicals' effect on the physiological dynamics of the cells, mechanistic modeling is required (Liang et al., 2014). Along these lines, Pan et al. (2013a,b) presented a two-exponent model for describing the cellular response towards toxic chemicals. More recently, Witzel et al. (2015) proposed a model for the growth curves of various colon cancer cells that allows predicting cell cycle time and IC_{50}. However, to the best of our knowledge, the aspect of mechanistic mathematical modeling has not yet been entirely explored with respect to predictive toxicology.

In this study, using the *in vitro* RTCA-based cell index data, we develop a methodology for quantifying the cytotoxicity of chemicals with known genotoxicity *in vivo*. As shown in Fig. 2, the RTCA-based cell index curves can be used to interpret the specific behavior of the cells (Kho et al., 2015). The initial period of the cell index curve illustrates the phase of cell adhesion and spreading (Fig. 2, left part). It is followed by a plateau phase prior to a gradual period of proliferation (Kho et al., 2015). Treatment with a chemical exposes the cells to an immediate and transient phase of genotoxic effects (Fig. 2, middle part), which denote damage to the DNA arising from the specific MoA of the chemical (Scott et al., 1991). During this transient delay, cell cycle checkpoints are elicited in response to chemical treatment that allows cells to repair damage before progressing to the next phase of the cycle (Shackelford et al., 1999). Subsequently, the DNA damage accumulated during the genotoxic phase manifests in the form of cytotoxicity (Fig. 2, right part) representing an overall decline in cell survival and proliferation rate (Scott et al., 1991). Along these lines, we propose that the profiles from the cytotoxic phase can be used as a basis for the identification of test concentrations that are relevant for predicting chemical's genotoxicity *in vitro* and possibly *in vivo*.

In the presented work, we develop and validate an analytical framework to investigate the *in vitro* RTCA data on cultured IEC-6 cells for predicting cytotoxicity. We first propose a qualitative analysis based on the area under the curve to assess the relevance of the data for characterizing the toxicity of test chemicals. Thereafter, based on the insights obtained from this pre-analysis, a kinetic model is introduced for predicting the temporal- and dose-dependent effects of chemicals in terms of its 50% inhibitory concentration IC_{50} and parameters related to cell turnover, taking the actual cell impedance dynamics into account. The IC_{50} values obtained using our approach are compared to the values obtained using a sigmoidal dose-response model described in the RTCA software (Manual, 2009). By presenting a foundation work for modeling and analyzing the RTCA datasets for predicting cytotoxicity, we believe that the study will contribute to the goal of improving *in vitro* strategies for genotoxicity testing (Waters and Fostel, 2004).

2. Methods

2.1. Chemicals

We evaluated 10 chemicals using a panel of test concentrations, summarized in Table 1. In general, we used chemicals that are recommended for evaluating the sensitivity and specificity of novel mammalian cell genotoxicity assays (Kirkland et al., 2008). Importantly, these chemicals have been characterized for *in vivo* genotoxicity in rodents, and provide the possibility of correlating *in vitro* findings with existing *in vivo* data. Chemicals possessing tissue-specific genotoxicity *in vivo* included 2-acetylamino-fluorene, aflatoxin B1, benzo-[a]-pyrene, chloramphenicol and N-ethyl-N-nitrosourea. On the other hand, erythromycin, urea, D-mannitol, resorcinol and sulfisoxazole comprised chemicals that have not been found to exhibit genotoxicity. Non-genotoxic controls like urea, erythromycin and sulfisoxazole have been reported to produce false-positive results in *in vitro* genotoxicity testing, despite clearly negative results *in vivo* (Kirkland et al., 2008). We chose to include these substances in order to account for the problem of false-positive testing.

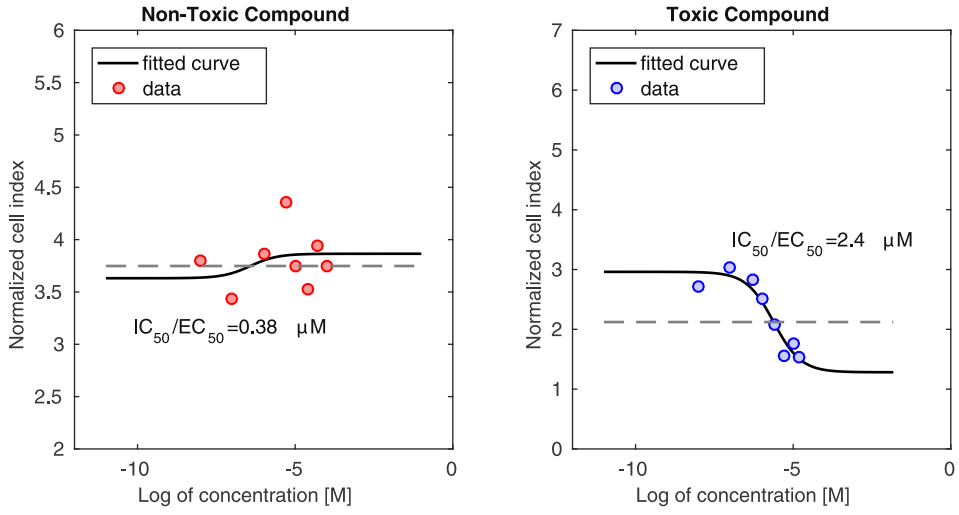

Fig. 1. Example fit. Figure illustrates the fit of a simulated experimental data set to the in-built sigmoidal dose-response model. We simulated data without and with effect plus white noise for the non-toxic and toxic compound labelled in red and blue, respectively. A low estimate of IC_{50}/EC_{50} for the non-toxic compound (left panel) shows that the model produces a false positive result, although the absolute magnitude of change in cell index is small.